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Deoxyribonucleic Acid, Theory of Techniques for Separation

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Introduction

Separation of biochemical molecules can be carried out in gels or polymer solutions and, in specific cases, in free solution, using constant or variable electric fields. Gels are used primarily in deep-dish containers, submerged in buffer, and polymer solutions are used in glass capillaries, with inner diameters less than 100 μm. Thin gels between two glass plates have been used for separating and sequencing single-stranded DNA molecules. We begin the theoretical discussion by considering the separation of double-stranded DNA molecules (dsDNA) in submarine gels under constant electric field conditions.

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Geometrical Sieving Model for Small DNA Molecules in a Constant Electric Field

Ogston was the first to calculate the fractional volume available to a sphere of radius R in a gel of a given concentration. The gel itself was modelled as a random array made up of fibres of radius r. Within this description, a sphere with a radius $R \gg r$ cannot pass through the network if the sphere is not allowed to deform. This geometrical model predicts that the electrophoretic mobility of DNA molecules, as a result of molecular 'sieving', varies as:

$$\frac{\mu}{\mu_0} \propto \exp\left[-\left(\frac{R_g}{a}\right)^2\right] \qquad [1]$$

where R_g is the radius of gyration of the DNA molecule, μ_0 is the free solution mobility, a is the average pore size of the gel, and the exponential dependence of the mobility arises from the assumption of Poisson statistics for the distribution of spaces in a random network of straight fibres. This model describes the mobility of small molecules when they first encounter the gel fibres as obstacles to molecular motion. The analysis of experimental data using eqn [1] is highly model dependent, but can provide some guidance for the development of new gel structures for more efficient electrophoretic separations of small molecules.

Entropic Trapping of Small DNA Molecules

For DNA in the entropic size regime, the deformable molecules select the larger pores in order to maximize locally their conformational entropy. However, in order to accomplish this, they must squeeze through the narrow channels connecting the larger pores. The corresponding polyelectrolyte dynamics is dominated by an activation process in this regime, where the electrophoretic mobility is given by an inverse power law (> 1) over a size range that is larger than for the Ogston regime, but smaller than for the beginning of reptation, which is discussed in the next section.

Gel Electrophoresis of Large DNA Molecules in a Constant Electric Field

Figure 1 shows a schematic picture of a gel matrix, in which a DNA molecule is embedded. For a molecule that is much longer than the average spacing between the chemical cross-links of the gel fibres, the molecule cannot move through the gel as a random coil, rather

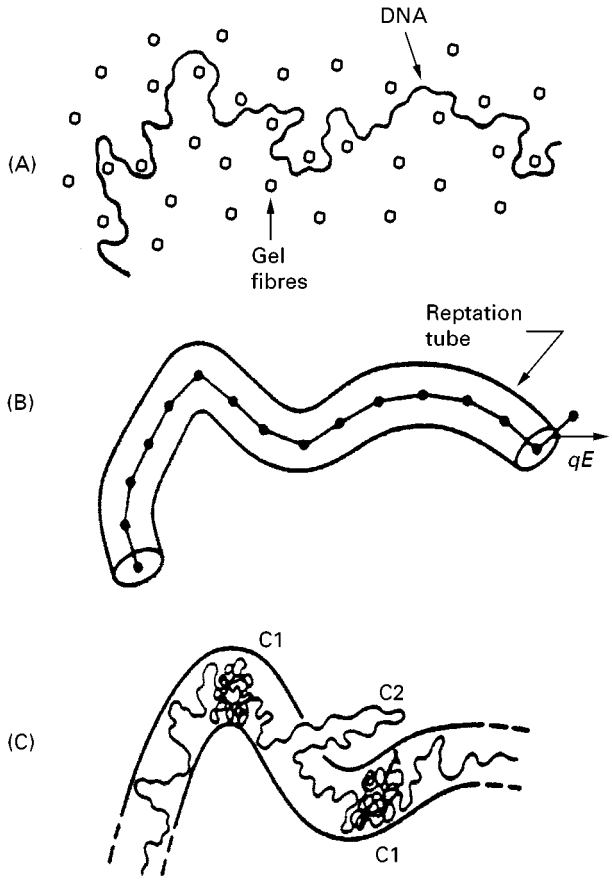

Figure 1 Schematic representation of a DNA molecule in a two-dimensional gel (A), in which the open dots represent obstacles corresponding to the gel fibres. (B) Shows how the obstacles hindering the motion of the molecule are approximated by a tube and the polymer by a chain of beads; the electric field exerts a force qE on the last bead and orients the segment leaving the tube. The tube is defined by the molecular conformation so that an extended conformation (with less DNA per gel pore) has a longer tube. (C) Charge gradients (C1) along the tube axis, and field-driven tube leakages (C2) are neglected in the biased reptation model.

it must reptate (from the Latin *reptare*, to creep) around the obstacles (cross-links), in a way that is analogous to the movement of polymer molecules in a self-entangled polymer melt. In this situation, the natural length scale is the average pore size of the gel, a. In terms of this length scale, we define a number of different quantities that arise in a theoretical description of the problem. One of these is the number of gel pore segments, N, occupied by the molecule:

$$N = \frac{M}{M_a} \qquad [2]$$

were M is the relative molecular mass of the molecule and M_a is the average relative molecular mass in a gel pore. Under the influence of an electric field, M_a can change as a function of time, depending on the stiffness of the molecule, the pore size, the magnitude of the electric field, and other factors. Another important quantity is the scaled effective electric field, E_{eff}:

$$E_{\text{eff}} = \frac{e_1 M_a E a}{2k_B T} = \frac{E}{E_a} \qquad [3]$$

which defines the intrinsic electric field parameter E_a. The net charge per nucleotide for DNA is denoted by e_1. This quantity depends on the native charge on the molecule, as well as on the charge screening properties of the buffer solution used for the electrophoretic separation. A simple way of understanding the limitations for separating large molecules in a gel using constant electric fields is as follows.

For constant a and M_a, the electrical force F_l on the molecule in the longitudinal direction (also known as the tube axis, if the molecule is considered to fill a 'tube' made up of occupied gel pores) is:

$$F_l = (q\boldsymbol{E}) \cdot \left(\sum_i \frac{\boldsymbol{r}_i}{a}\right) \qquad [4]$$

where $q = e_l M_a$ is the average net charge of the molecule in a gel pore, and $\boldsymbol{r}_l/a$ is the unit vector pointing from pore i to pore $i + 1$ along the molecule, where the dot in eqn [4] indicates the dot product of two vectors. This expression reduces to:

$$F_l = qE\frac{h_x}{a} \qquad [5]$$

where h_x is the end-to-end distance of the molecule in the field direction. The force is time-dependent and fluctuates during electrophoretic migration. Opposing the migration along the tube is a friction coefficient $\xi = \xi_0 N$, where ξ_0 is the friction coefficient per tube segment, as defined by eqn [2]. The instantaneous velocity of the chain along the longitudinal axis is then:

$$v_l = \frac{F_l}{\xi} = \left(\frac{qE}{\xi}\right)\left(\frac{h_x}{a}\right) \qquad [6]$$

and the average mobility of the centre of mass (defined as the velocity per unit electric field in the field direction) is given by:

$$\mu = \frac{\langle v_l(h_x/N)\rangle}{E} = \mu_0 \frac{\langle h_x^2\rangle}{N^2} \qquad [7]$$

where $\mu_0 = q/\xi_0 a$, and the geometrical factor (h_x/N) in eqn [7] takes into account the fact that the vector

$\boldsymbol{h}$ is in general not parallel to the direction of the electric field. For small electric fields and/or small molecules, $\langle h_x^2 \rangle$ proportional to N, giving μ proportional to $1/N$. In this regime the molecules reptate while retaining their random-walk conformations. For large molecules and/or high electric fields, the molecules become stretched in the electric field direction during migration, and $\langle h_x^2 \rangle$ is proportional to N^2, resulting in a mobility that is independent of the molecular mass, according to eqn [7].

Figure 2 shows a log–log plot of the reduced electrophoretic mobility, μ/μ_0, from Slater and co-workers as a function of the scaled molecular size, $N = M/M_a$, for different values of the scaled electric field. From top to bottom, $E_{\text{eff}} = 1.0$, 0.2, 0.1, 0.01. For small molecular sizes, the mobility is independent of the field intensity and deceases as $1/N$. For large molecular sizes, the mobility is independent of size and increases as E_{eff}^2. The small minimum in the mobility is a phenomenon known as band inversion, for which, in a limited size range, large molecules can move faster than smaller molecules. Noolandi and co-workers have shown that this is a statistical effect where linear molecules, which have two free ends, can migrate into slowly moving 'J' or 'U' states for different time intervals, depending on the specific electrophoresis conditions. Since the bands of DNA molecules are not in order of increasing size in this region, one has to be careful not to mislabel the molecular fragments.

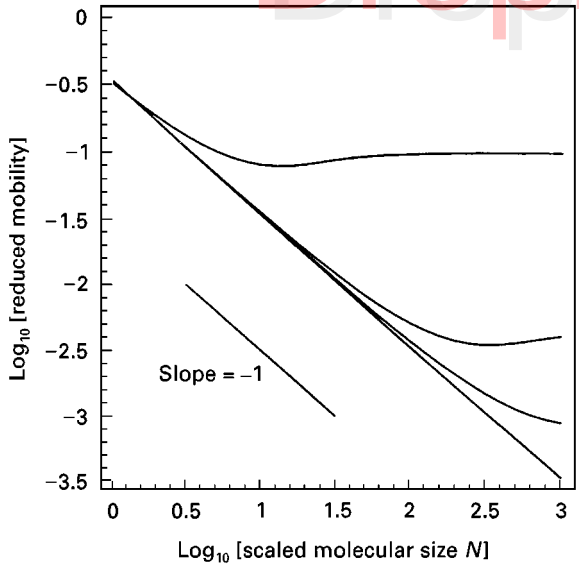

Figure 2 Log–log plot of the reduced electrophoretic mobility μ/μ_0 as a function of the scaled molecular size $N = M/M_a$ for different values of the scaled constant electric field E_{eff} (see text). For small molecular sizes the mobility is independent of the constant field intensity and decreases with size as $1/N$. For large molecular sizes, the mobility is independent of size and increases as E_{eff}^2. The electrophoretic mobility has a shallow minimum for intermediate molecular sizes.

In summary, the basic reason for the constant plateau mobility in Figure 2 is that for stretched molecules the electrical driving force and the opposing friction both scale linearly with length, and the resulting ratio is independent of length. For freely draining molecular coils in free solution, the same effect occurs, with the result that it is not possible to separate freely draining polyelectrolytes according to size in free solution electrophoresis. However, there are ways to overcome this limitation at least partly for free solution electrophoresis, as we discuss later. Next we turn to overcoming the limitations of constant field gel electrophoresis.

Pulsed Field Gel Electrophoresis of Large DNA Molecules

In constant field gel electrophoresis the maximum size of dsDNA that can be separated is about 50 kilobases. In pulsed-field gel electrophoresis separations up to a few megabases can be achieved. In this section we explain why such an increase in separation latitude takes place.

The plateau mobility is reached because of molecular stretching in a constant electric field. Schwartz and Cantor showed that the way to avoid this is to change the electric field constantly, either in magnitude and/or direction. The disadvantage of doing this is that it can take a long time to separate large molecules (separating dsDNA molecules a few megabases in size can take a few days). The advantage, of course, is that it is possible to separate large molecules at all, provided that the variations in the electric field are chosen properly. Changes in the magnitude and/or direction of the electric field force the molecules to adapt to the new electrophoresis conditions. However the adaptation time (also known as the 'relaxation' time) is very specific to the molecular size, average gel pore diameter, and electric field changes. It follows that there are a large number of ways to implement this process. Some pulsed-field gel separations use electric fields of the same magnitude and only change the field direction in two dimensions. Others keep the electric field in one dimension (forwards and backwards) for different time periods and with different amplitudes). We use the one-dimensional case developed by Turmel and co-workers as an example of how separations can be carried out by tuning the pulse times to the relaxation times of the molecules in the gel.

Figure 3 is a schematic illustration of the displacement of two types of molecules, molecular masses

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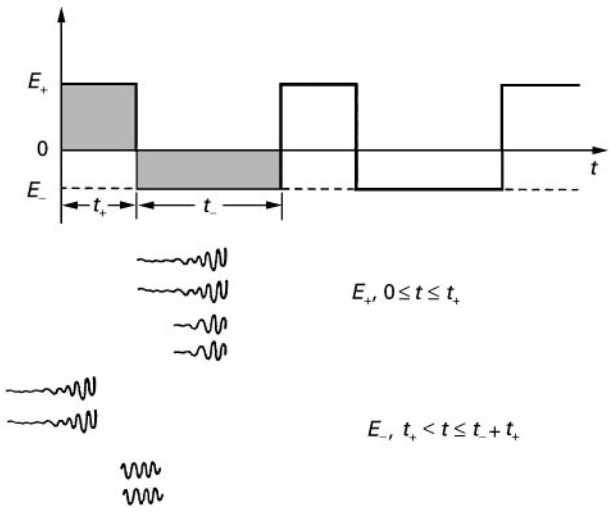

Figure 3 Pulse shape for gel separations of large DNA molecules, using zero-integrated-field electrophoresis (ZIFE). As explained in the text, this pulse shape allows the proper size-migration distance to be maintained at all times, and gives rise to a sharp drop in the electrophoresis mobility as a function of size for fixed pulse parameters. For given electric field amplitudes, the reverse pulse time, t_-, defines the relaxation time of the largest molecule that can adapt to the lower electric field and have a net forward displacement during one complete cycle.

M_1 and M_2 ($M_1 < M_2$), when subjected to a high field forward pulse, followed by a low field backward pulse of longer duration than the forward pulse. During the pulse duration t_+, with field intensity E_+, the displacements of molecules M_1 and M_2 are $\mu(E_+, M_1)E_+t_+$ and $\mu(E_+, M_2)E_+t_+$ respectively, where the dependence of the mobilities on the field strength and molecular size has been explicitly indicated. During the reverse pulse of duration t_-, with absolute field intensity E_-, the reverse displacement of the shorter molecule is $-\mu(E_-, M_1)E_-t_-$, if t_- is long enough to allow the shorter molecule to relax to the new electric field in the backward direction. The net displacement of the shorter molecules over the time period $t_+ + t_-$ is then $\mu(E_+, M_1)E_+t_+ - \mu(E_-, M_1)E_-t_-$. However, for the longer molecule M_2, the reverse displacement is $-\mu(E_+, M_2)E_-t_-$, if the time interval t_- is too short to allow the longer molecule relax to the lower electric field intensity E_- in the reverse direction. The net displacement of the longer molecule is then $\mu(E_+, M_2)E_+t_+ - \mu(E_+, M_2)E_-t_-$, and vanishes when $E_+t_+ = E_-t_-$. This is known as the zero-integrated field condition. With this condition the displacement of the shorter molecule becomes $[\mu(E_+, M_1) - \mu(E_-, M_1)]E_+t_+$, which is in the forward direction since $\mu(E_-, M_1) < \mu(E_+, M_1)$ if $E_- < E_+$. The zero-integrated pulse form allows the proper molecular size versus displacement in the gel to be maintained at all times, avoiding the problem of band inversion. Although the tight DNA bands achieved by this process is an advantage, the running time for experiments is longer than for some other pulse schemes. Its usefulness depends on the specific requirements of the practitioner. The pulse times are determined experimentally using known molecular size markers, which show a sharp drop in mobility when the relaxation times are reached for a given size range. Using this information, a general electric field pulse scheme can be programmed for separating unknown DNA size distributions.

Capillary Zone Electrophoresis (CZE)

Capillary electrophoresis of DNA is a specific example of a technique used to separate molecules known as capillary zone electrophoresis (CZE), in which fused silica capillaries have their ends inserted in electrolyte reservoirs which also contain electrodes. This powerful analytical technique, as discussed by Gordon and co-workers, has been developed over the past decade in a number of different academic and industrial laboratories. Here the walls of the fused silica capillaries have a negative charge resulting from the ionization of the surface silanol groups in aqueous solution. When a potential difference is established between the electrodes, a bulk flow of fluid towards the cathode takes place. This is called electroosmotic flow, and results from the electrical double layer formed at the wall–electrolyte interface. The electroosmotic velocity is given by:

$$V_{eo} = \mu_{eo}E = \frac{\varepsilon\varepsilon_0\zeta E}{\eta} \qquad [8]$$

where $\zeta = \delta\sigma/\varepsilon_0$, and μ_{eo} is the electroosmotic mobility, E is the electrical field, ε_0 the permittivity of the vacuum, ε the relative permittivity, ζ the zeta potential, η the viscosity, δ the double layer thickness and σ the surface excess charge density. The double layer thickness is inversely proportional to the square root of the molar concentration of the buffer solution. The electroosmotic flow enables the separation of charged molecules according to their different electrophoretic mobilities. In this method, positively charged molecules are eluted first because their electrophoretic motion and the electroosmotic motion are in the same direction. Negatively charged molecules try to move in the opposite direction, but if their electrophoretic mobility is less than the electroosmotic mobility, the net result is that they are eluted later than the positive molecules. Thus electroosmotic flow can be considered as a built-in pump which is useful for carrying out electrokinetic separations.

Clearly this process cannot separate neutral molecules if only an electrolyte is used. Sometimes micelles are dissolved in the separation electrolyte, resulting in some separation of uncharged species because of their partitioning between the micelles and the electrolyte. More commonly, one packs the capillary with a stationary phase, which can be bonded to the walls of the capillary, or formed by close packed small particles. The flow profile in the capillary has a nearly flat (plug-like) profile instead of the usual parabolic profile for Poiseuille flow when a viscous, incompressible fluid is pushed through a cylindrical tube by a pressure difference. The nearly flat flow profile makes the separation and detection of small amounts of analyte easier. This technique is known as capillary electrochromatography (CEC). The packing of the capillaries can be carried out electrokinetically, using commercially available small (a few μm) porous silica particles.

A number of different theoretical strategies have been used to increase the sensitivity of analyte detection. Programming of the electric field to achieve uniform sensitivity for on-line detection has been used, and theories for maximizing the signal-to-noise ratio for the case of laser-induced fluorescence and UV absorbance detection in capillary electrophoresis have been developed. In particular, for rather general conditions, the ratio of the number of fluorescent molecules in two analyte bands can be shown to be proportional to the ratio of the heights of the band peaks. This is useful for fluorescent analytes, and for cases where nonfluorescent analytes are labelled with fluorescent probes prior to detection.

Capillary Electrophoresis of DNA Molecules in Free Solution

In free solution the electrophoretic mobility of a free draining polyelectrolyte coil is equal to the ratio of its electric charge to its friction coefficient. Since both quantities scale linearly with molecular mass, the mobility is independent of molecular size. Hence the only way to separate molecules of different size in this case is to break the scaling symmetry of the charge and/or friction with size. As shown by Voelkel and Noolandi, adding a molecular unit with a different charge and friction to one of the ends of a linear polyelectrolyte, such as DNA, will change the free solution mobility to:

$$\mu(M) = \mu_0 \frac{M + q_{\text{eff}}}{M + \xi_{\text{eff}}} \qquad [9]$$

where μ_0 is the free solution mobility of the unlabelled free draining polyelectrolyte, q_{eff} is the ratio of the effective charge carried by the label expressed in terms of the charge per monomer unit of the polyelectrolyte, and ξ_{eff} is the friction of the label expressed in terms of the friction per monomer unit of the polyelectrolyte. In this approximation the hydrodynamic interaction of the label and the polyelectrolyte is neglected. The free solution mobility is now a function of the molecular mass. However the effectiveness of this strategy depends on the size of the label, compared with the rest of the molecule, and the amount of the band broadening caused by Brownian motion and other effects. In practice this approach seems viable for separating single-stranded DNA molecules up to several hundred bases long for DNA sequencing applications. While gel separations of single-stranded DNA molecules can routinely be carried out for sizes well over 500 bases, free solution separations, which are only effective for shorter fragments, can be carried out only an order of magnitude faster.

Capillary Electrophoresis of DNA Molecules Using Dilute Polymer Solutions

A separation mechanism that combines aspects of both free solution and gel electrophoresis is separation in capillaries using ultrathin polymer solutions. Here the DNA molecules drag along the polymer molecules that they encounter during migration. The capture and release of the unentangled polymer molecules results in the separation of DNA molecules. This new type of separation mechanism, developed by Barron and co-workers, is based on hydrodynamics, drag forces and molecular collisions, and is best suited for high throughput applications. The theoretical basis for the separation is still under development.

Capillary Electrophoresis of DNA Molecules Using Entangled Polymer Solutions

As pointed out by Slater, entangled solutions, as opposed to a gel, involve physical, instead of chemical, cross-links. For polymer solutions, we consider concentrations $c > c^*$, where c^* is called the critical concentration for entanglements:

$$c^* = \frac{M}{\left(\frac{4\pi}{3} R_g^3\right)} \qquad [10]$$

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M is the relative molecular mass, and R_g is the radius of gyration of the polymer molecule. For $c > c^*$ the polymer coils overlap and a loosely associated polymer network is formed. From the theory of polymer networks in solution we can characterize the mean pore size by a 'blob' dimension:

$$\xi = 1.4R_g\left(\frac{c^*}{c}\right)^{3/4} \quad [11]$$

However we must bear in mind that we are dealing with loosely associated networks, and that the physical cross-links are temporary, as opposed to chemically cross-linked networks. As discussed by Viovy and Heller, for low electrophoretic mobilities the dissociation and reassociation times of the network become important, because they occur on the same timescale as the transit time of the DNA molecules through the network. As a consequence, it is not possible to achieve better separations of DNA molecules through a concentrated polymer solution than through a gel, however polymer solutions are more easily processed (injected or removed) through capillary channels than cross-linked gels because of their low viscosities. Some polymer solutions have strongly temperature-dependent properties, which allows more latitude in the processing conditions.

Future Developments

Separations of biomolecules are being carried out on smaller and smaller devices, using miniaturized fluid-handling devices and detection systems. Making use of recent rapid developments in the area of microelectromechanical systems (MEMS), which exploits advances in microlithography for the semiconductor industry, new biochips and biosensors have been designed that enable faster analytical and diagnostic techniques to be carried out than was possible with macroscopic devices. As shown by Chee and coworkers, hundreds of thousands of DNA oligonucleotide probes can be assembled on a glass microchip, and combined with micromachined capillary electrophoresis injectors and separators. Complete hybridization patterns can be revealed in a matter of minutes, using laser-induced fluorescence. The implications for the entire biotechnology industry are revolutionary. Coupled with the knowledge obtained from the Human Genome Program, in which the estimated 100 000 human genes are in the process of being discovered and sequenced, thousands of genetic variations can be analysed in a single experiment, making possible the rapid localization of disease-causing genes.

From the theoretical point of view, there are several areas where advances are necessary to keep up with the rapid developments in instrumentation. First, new software codes are required to enable the rapid deciphering and processing of the massive amounts of bioinformation that are being generated. Second, detection systems with higher resolution than is currently available must be designed to interpret the spectral data that are available with the use of laser-induced fluorescence. Finally, theoretical modelling of the behaviour of electrolytes and biomolecules in microchannels in the presence of electric fields will be useful in understanding the ultimate capability of microdevices for the applications that we have mentioned.

See Colour Plate 40.

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